IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:Confirmation No. 3250
Serial No. 10/591,752	: Group Art Unit 1638
Akiho Yokota et al.	: Attorney Docket No. 2006_1303A
Filed : September 26, 2006	: Examiner PAGE, BRENT T

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks

Sir:

I, Akiho Yokota hereby declare that:

I was born in Kagawa prefecture, Japan, in 1948;

I am a citizen of Japan and a resident of 108-37-11, Nishimatsugaoka Ikoma-shi, Nara 630-0246 JAPAN;

I graduated from Department of polymer chemistry, Faculty of agricuture, Osaka Prefectural University, Japan in 1977.

I received my doctor degree on the study of "Physiological analyses of subcellular organelles including mitochondria and microbodies in Euglena gracilis" at Osaka Prefectural University, Osaka, Japan, in 1978;

I have worked as a Professor of Nara Institute of Science and Technology, National University Corporation in Japan from

1996 until now and have engaged in a study on Plant Molecular Biology and Engineering;

I am one of the inventors for this application;

I have many reports relating to study of molecular mechanism and genetical evolution on photosynthesis in plants and algae. The reports are as follows:

1: Nakano T, Ashida H, Mizohata E, Matsumura H, Yokota A. An evolutionally conserved Lys122 is essential for function in Rhodospirillum rubrum bona fide RuBisCO and Bacillus subtilis RuBisCO-like protein. Biochem Biophys Res Commun. 2010 Jan 11. [Available online]

2: Saito Y, Ashida H, Sakiyama T, de Marsac NT, Danchin A, Sekowska A, Yokota A. Structural and functional similarities between a ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)-like protein from Bacillus subtilis and photosynthetic RuBisCO. J Biol Chem. 2009 May 8;284(19):13256-64.

3: Yabuta Y, Tamoi M, Yamamoto K, Tomizawa K, Yokota A, Shigeoka S. Molecular design of photosynthesis-elevated chloroplasts for mass accumulation of a foreign protein. Plant Cell Physiol. 2008 Mar;49(3):375-85.

4: Carré-Mlouka A, Méjean A, Quillardet P, Ashida H, Saito Y, Yokota A, Callebaut I, Sekowska A, Dittmann E, Bouchier C, de Marsac NT. A new rubisco-like protein coexists with a photosynthetic rubisco in the planktonic cyanobacteria Microcystis. J Biol Chem. 2006 Aug 25;281(34):24462-71.

5: Kato Y, Murakami S, Yamamoto Y, Chatani H, Kondo Y, Nakano T, Yokota A, Sato F. The DNA-binding protease, CND41, and the degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase in senescent leaves of tobacco. Planta. 2004 Nov;220(1):97-104.

6: Ashida H, Saito Y, Kojima C, Kobayashi K, Ogasawara N, Yokota A. A functional link between RuBisCO-like protein of Bacillus and photosynthetic RuBisCO. Science. 2003 Oct 10;302(5643):286-90.

7: Nakahara K, Yamamoto H, Miyake C, Yokota A. Purification and characterization of class-I and class-II fructose-1,6-bisphosphate aldolases from the cyanobacterium Synechocystis sp. PCC 6803. Plant Cell Physiol. 2003 Mar;44(3):326-33.

8: Mizohata E, Matsumura H, Okano Y, Kumei M, Takuma H, Onodera J, Kato K, Shibata N, Inoue T, Yokota A, Kai Y. Crystal structure of activated ribulose-1,5-bisphosphate carboxylase/oxygenase from green alga Chlamydomonas reinhardtii complexed with 2-carboxyarabinitol-1,5-bisphosphate. J Mol Biol. 2002 Feb 22;316(3):679-91.

9: Osafune T, Yokota A, Sumida S, Hase E. Immunogold Localization of Ribulose-1,5-Bisphosphate Carboxylase with Reference to Pyrenoid Morphology in Chloroplasts of Synchronized Euglena gracilis Cells. Plant Physiol. 1990 Mar;92(3):802-808.

10: Yokota A, Canvin DT. Changes of Ribulose Bisphosphate Carboxylase/Oxygenase Content, Ribulose Bisphosphate Concentration, and Photosynthetic Activity during Adaptation of High-CO(2) Grown Cells to Low-CO(2) Conditions in Chlorella pyrenoidosa. Plant Physiol. 1986 Feb;80(2):341-345.

11: Yokota A, Canvin DT. Ribulose Bisphosphate Carboxylase/Oxygenase Content Determined with [C]Carboxypentitol Bisphosphate in Plants and Algae. Plant Physiol. 1985 Mar;77(3):735-739.

The experiments given below were conducted under my supervision.

Experiment

(1)Purpose

The experiments were designed to compare the photosynthetic activity and FBPase activity of a plant transformed with the vector of the present invention with those of a plant transformed with the vector taught by Yokota.

(2)Method

(2-1)Preparation of transformants

Example

[Step 1] Preparation of pLD6-S.7942FBP/SBPase

A Synechococcus PCC 7942 derived-FBP/SBPase gene (fbp/sbp) was inserted between restriction enzymes SphI and EcoRI sites of a vector pLD6 having the psbA promoter (PpsbA) by which high expression can be expected in tobacco chloroplasts, to prepare pLD6-S.7942FBP/SBPase. This pLD6-S.7942FBP/SBPase was introduced into Escherichia coli according to a conventional method. This Escherichia coli was cultured at 37°C for 16 hours in LB medium supplemented with spectinomycin to select the Escherichia coli in which such gene was introduced. The selected Escherichia coli was cultured under the similar condition, cells were collected by centrifugation, and pLD6-S.7942FBP/SBPase (plasmid DNA) was purified by a conventional method. The LB medium includes 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter.

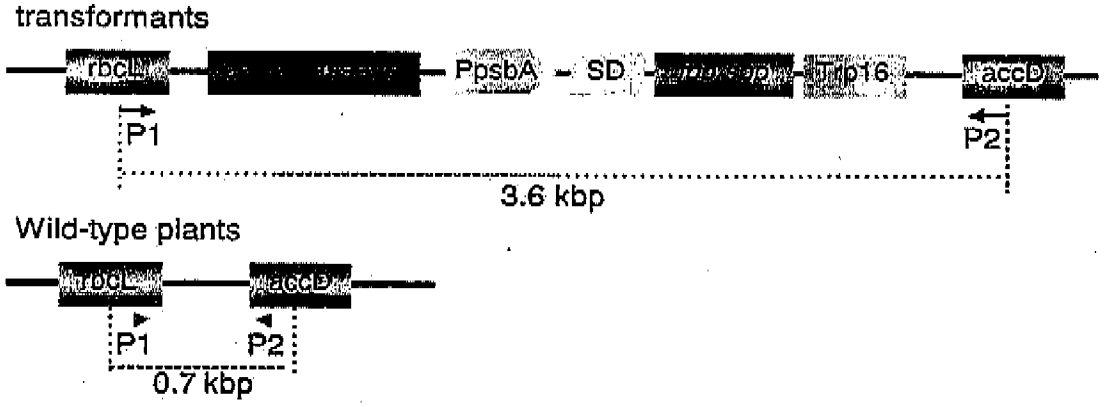
[Step 2] Preparation of pLD200-S.7942FBP/SBPase

The pLD6-S.7942FBP/SBPase purified in the step 1 was treated with restriction enzymes NotI and SalI, and then the fragment containing S.7942FBP/SBPase was inserted between NotI and SalI sites of the vector pLD200 for transforming chloroplasts which contains a part of the rbcL gene and a part of the accD gene of the tobacco chloroplast genome upstream of

NotI and downstream of SalI, to prepare pLD200-S.7942FBP/SBPase. This pLD200-S.7942FBP/SBPase was introduced into Escherichia coli according to a conventional method. This Escherichia coli was cultured at 37°C for 16 hours in LB medium supplemented with spectinomycin to select the Escherichia coli in which such gene was introduced. The selected Escherichia coli was cultured under the similar condition, cells were collected by centrifugation, and pLD200-S.7942FBP/SBPase (plasmid DNA) was purified according to a conventional method.

Construction of the obtained plasmid is shown in Fig.1 below.

Fig.1



[Step 3] Preparation of chloroplast transformant

The purified pLD200-S.7942FBP/SBPase was introduced into tobacco chloroplasts with a particle gun to prepare a chloroplast transformant. The transformation of tobacco chloroplasts was carried out according to the known method (Svab,Z., Hajdukiewicz,P. and maliga,P., Stable transformation of plastids in higher plants. Proc.Natl.Acad.Sci.USA, 87, 8526-8530(1990)).

After redifferentiation on a spectinomycin-supplemented medium, a transformant (pTpsbAFS) 6 strain wherein S.7942FBP/SBPase was introduced into the chloroplast genome could be obtained by PCR. Also in T_1 generation produced by self hybridization, defect of the gene was not recognized.

Western blotting was performed using an anti-S.7942FBP/SBPase antibody and, as a result, the signal was

recognized at a position of about 40kDa coinciding with a molecular mass of S.7942FBP/SBPase only in the transformed plant (pTpsbAFS), and it was made clear that FBP/SBPase was highly expressed.

Among the 6 transformant strains, 2 strains(pTpsbAFS-3 and 9) were used in measurement of photosynthesis activity, and 4 strains(pTpsbAFS-1, 2, 9 and 11) were used in measurement of FBPase activity.

Comparative Example

Tomato rbcS promoter, coding region of a transit peptide and Synechococcus PCC 7942 derived-FBP/SBPase gene (fbp/sbp) were conjugated with pBI101 to construct a plasmid. The plasmid was incorporated into agrobacterium tumefacience LBA4404, which was used for infection of leaf disk of tobacco(Nicotiana tabacum cv Xanthi), to incorporate fbp-1 into a tobacco nuclear gene. After isolating the genomic DNA, incorporation of fbp-1 was confirmed by PCR and immunoblotting methods. 7 strains of transformants(TFI-1 to TFI-7) were obtained . The chloroplasts were isolated from transformed strains(T2 generation), then expression of S.7942 FBPase/SBPase was confirmed by western blotting. Moreover, it was confirmed, by cell fractionation, that the protein expressed from the incorporated gene is localized in the chloroplasts.

Among those 7 transformant strains, 4 strains(TpFS-1, 3, 4 and 6) were used in measuring phtotosynthesis activity, and 2 strains (TpFS-3 and TpFS-6) were used in measuring FBPase activity.

(2-2) Measurement of photosynthesis activity

Using a T_1 generation 12 weeks after seeding of transformants of Example and Comparative example, photosynthesis activity was measured by a change in light intensity under condition of the CO_2 concentration of 360 ppm.

(2-3) Measurement of FBPase activity

FBPase was assayed by monitoring the reduction of $NADP^+$ at 340 nm according to the method of Zimmermann et al. (Eur. J. Biochem. (1976) 253, pp. 5952-5956). In the final volume of

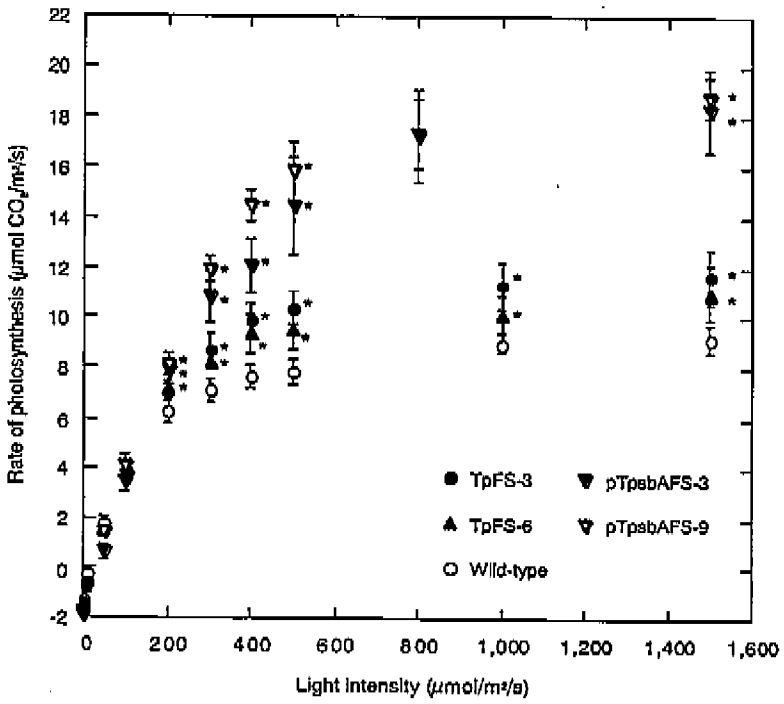
1 ml, the reaction mixture contained 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.4 mm $NADP^+$, 0.1 mM Fru 1,6-P_2, 0.5 units of glucose-6-phosphate dehydrogenase, 1.5 units of phosphoglucose isomerase, and the enzyme. The reaction was started by addition of Fru 1,6-P2 after preincubation for 2 min.

(3)Results

(3-1) Photosynthesis activity

Results are shown in Fig. 2 below.

Fig.2



The photosynthesis rate of the transformants of Example(pTpsbAFS-3 and pTpsbAFS-9)at a maximum was about 2-fold that of the wild strain.

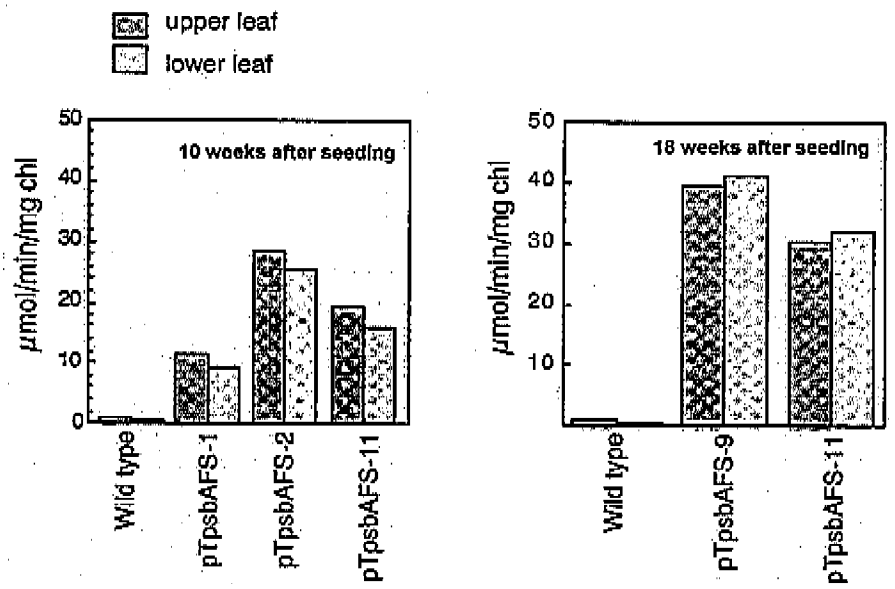
On the other hand, the transformants of Comparative Example(TpFS-3 and TpFS-6) had an about 1.2 to 1.3-fold photosynthesis rate at a maximum as compared with a wild strain.

The transformants of Example were 1.5 to 1.7-fold superior to those of Comparative Example in photosynthesis activity.

(3-2) FBPase activity

Result of Example is shown in Fig.3 below. FBPase activity of the transformants of Example (pTpsbAFS-1, 2, 9 and 11) were about 10-40 μmol/min/mg chlorophyl. This activity is about 10 to 40-fold higher than that of the wild strain.

Fig.3



On the other hand, FBPase activity of the transformants of Comparative Example (TpFS-3 and TpFS-6) were about 1.82 ±0.24 μmol/min/mg chlorophyl, while FBPase activity of the wild type starin is 1.04±0.22μmol/min/mg chlorophyl. The FBBase activity of the transformants are 1.75 times higher than that of the wild type strain.

The transformants of Example were 6 to 23-fold superior to those of Comparative Example in FBPase activity.

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It is declared by the undersigned that all statements made herein of undersigned's own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the

knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18U.S.C.1001, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Date: 29/01/10

Akiho Yokota